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Discovery and characterization of dual inhibitors of MDM2 and NFAT1 for pancreatic cancer therapy

Wei Wang^{#1,2,*}, Jiang-Jiang Qin^{#1}, Sukesh Voruganti³, Bhavitavya Nijampatnam⁴,
Sadanandan E. Velu⁴, Ke-He Ruan¹, Ming Hu¹, Jianwei Zhou⁵, and Ruiwen Zhang^{1,2,*}

¹Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, Texas 77204, USA

²Center for Drug Discovery, University of Houston, Houston, Texas 77204, USA

³Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, TX 79106, USA

⁴Department of Chemistry, University of Alabama at Birmingham, Birmingham, AL 35294, USA

⁵Department of Molecular Cell Biology and Toxicology, Cancer Center, School of Public Health, Nanjing Medical University, Nanjing, P. R. China

These authors contributed equally to this work.

Abstract

Overexpression and activation of the MDM2 or NFAT1 oncogenes frequently occur in pancreatic cancer. Most MDM2 inhibitors under development target MDM2-p53 binding and have little or no effect on cancers without functional p53, including pancreatic cancer. Some available compounds indirectly inhibit NFAT1 activity by interfering with calcineurin activity, but there are currently no specific inhibitors against NFAT1. Here we performed a high-throughput virtual and cell-based screening to yield a lead compound (MA242) that can directly bind both MDM2 and NFAT1 with high affinity, induce their protein degradation, and inhibit NFAT1-mediated transcription of MDM2. As a result of this binding, MA242 decreased cell proliferation and induced apoptosis in pancreatic cancer cell lines regardless of p53 status. MA242 alone or in combination with gemcitabine inhibited pancreatic tumor growth and metastasis without any host toxicity. Our data indicate that targeting both MDM2 and NFAT1 represents a novel and effective strategy to treat pancreatic cancer.

Keywords

MDM2; NFAT1; p53-independent; CRISPR/Cas9; Pancreatic Cancer

*Correspondence to Dr. Ruiwen Zhang, MD, PhD, DABT, Department of Pharmacological and Pharmaceutical Sciences, School of Pharmacy, University of Houston, 4849 Calhoun Rd. Houston, Texas 77204, USA. rzhang27@central.uh.edu; Tel: 713-743-6840; Fax: 713-743-1229; Dr. Wei Wang, MD, PhD, Department of Pharmacological and Pharmaceutical Sciences, School of Pharmacy, University of Houston, 4849 Calhoun Rd. Houston, Texas 77204, USA. wwang4@central.uh.edu; Tel: 713-743-7349; Fax: 713-743-1229.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Introduction

Pancreatic cancer is a highly aggressive and commonly fatal malignancy which is characterized by early metastasis and a poor response to chemotherapy (1, 2). Although it demonstrates only modest clinical benefit, gemcitabine remains one of the mainstays of treatment for advanced pancreatic cancer (1). Various multidrug regimens that combine gemcitabine with other chemotherapeutic or molecular targeted agents have been evaluated in the preclinical and clinical settings. However, only three combination regimens have been approved by the United States Food and Drug Administration, and most of them failed to demonstrate statistically significant benefits on the survival of patients with pancreatic cancer in phase III clinical trials (3–5). Stromal depletion and immunotherapy have also been expected to offer substantial promise for treating advanced pancreatic cancer, but their therapeutic impact remains unclear (6). Thus, there is an unmet clinical need for novel, effective, and safe drugs for pancreatic cancer therapy.

The murine double minute 2 (MDM2) protein is overexpressed in a number of human malignancies, including pancreatic cancer (7, 8). MDM2 exerts its oncogenic activity via both p53-dependent and p53-independent pathways, promoting cancer cell growth and invasion, suppressing apoptosis, and inducing resistance to chemotherapy (7–12). MDM2 overexpression is associated with invasive and high grade/late stage tumors, metastasis, and recurrence, and is a negative prognostic factor for cancer patients with pancreatic cancer (7, 8). Recent advances in MDM2 biology have demonstrated that MDM2 is a valid target for cancer therapy (7, 8, 13, 14), and numerous studies using different types of MDM2 inhibitors have shown that inhibition of MDM2 can lead to decreased tumor growth and progression, and an improved response to conventional cancer therapies. Many MDM2 inhibitors have been developed to inhibit the MDM2-p53 interaction, MDM2 expression or its E3 ligase activity, and a few candidates have entered clinical trials. However, none of these inhibitors have been approved for clinical use (12, 14). Of note, almost all of the small molecule inhibitors (SMIs) of MDM2 that have been (or are being) tested in clinical trials target the binding of MDM2 and p53, and exert their anticancer effects primarily by activating the p53 pathway in cells with wild-type p53. Considering that these MDM2 SMIs require wild type p53 expression in cancer cells, they would be expected to have little or no activity against cancers with p53 deficiency or mutations. Unfortunately, genetic alterations of p53 are common in pancreatic cancer, and such cancers are more aggressive and more likely to metastasize (15, 16). Therefore, there is an urgent need to identify a novel, p53-independent strategy to inhibit MDM2.

Nuclear Factor of Activated T cells 1 (NFAT1) is the first identified member of the NFAT family. It has well-documented roles in the immune system and inflammatory response (17, 18), but its functions in cancer development and progression are still being elucidated (19, 20). NFAT1 promotes cancer cell proliferation, enhances cell cycle progression, suppresses apoptosis, induces migration and invasion, and leads to drug resistance through both calcineurin-dependent and -independent pathways (19–22). Therefore, we and others have proposed targeting NFAT1 as a novel anticancer strategy (19, 20). Some compounds have been found to inhibit NFAT1 activity by interfering with the calcineurin activity, but so far, there have been no specific NFAT1 inhibitors developed for cancer therapy (19).

In addition to the individual oncogenic properties of MDM2 and NFAT1, we have recently found that NFAT1 is a novel regulator of the MDM2 oncogene (23). NFAT1 directly binds to the MDM2 P2 promoter, promoting MDM2 transcription (23). Since both MDM2 and NFAT1 have independent oncogenic roles in pancreatic cancer, and based on the identification of the NFAT1-MDM2 pathway, a strategy targeting both MDM2 and NFAT1 may provide a novel treatment for pancreatic cancer. We herein report the identification of MA242 (Figure 1A), a small molecule that inhibits both MDM2 and NFAT1. The goals of this study were to demonstrate the therapeutic efficacy and safety of MA242 alone and in combination with gemcitabine, and to confirm its mechanisms of action in clinically-relevant models of pancreatic cancer, especially in those deficient in functional p53.

Materials and methods

Chemicals, plasmids, siRNA, CRISPR/Cas9, cell lines, and other reagents.

MA242 was synthesized and purified in Dr. Sadanandan E. Velu's laboratory (University of Alabama at Birmingham, AL). The MDM2 siRNA and control siRNA were from Thermo Scientific (Rockford, IL). For CRISPR/Cas9-mediated MDM2 knockout, the primer sequence of sgRNA-MDM2 was 5'-GTTGGGCCCCTTCGTGAGAAT-3'. The primer sequences used for T7EN1 assays and sequencing were as follows: MDM2-clev-forward, 5'-TGCTAGCATTCCTGTGACTGAG-3'; MDM2-clev-reverse, 5'-AAAGCCCCTCTTCAGCTTGTGT-3'. A plasmid construct with the GFP-targeted protospacer sequence was used as the negative control. All specific target sequences were amplified and cloned into lenti-CRISPR vectors and verified by DNA sequencing. Antibodies and plasmids were obtained commercially or were provided by other investigators. A detailed list is provided in the *Supplemental Methods*.

The human pancreatic cancer HPAC, Panc-1, AsPC-1, Mia-Paca-2 and BxPC-3 cell lines were obtained from the American Type Culture Collection (Rockville, MD). The human pancreatic ductal epithelium (HPDE) cell line was a kind gift from Dr. M.S. Tsao (University of Toronto, ON, Canada). Cell lines included in the study were validated by analysis of STRs (GenePrint® 10 System, Promega) and checked for mycoplasma contamination by PCR.

Assays for the *in vitro* anticancer activity of MA242.

All of the *in vitro* assays used to determine the effects of MA242 alone or combined with gemcitabine on cell viability (24, 25), colony formation (24, 25), cell apoptosis (24, 25), cell cycle distribution (24, 25), cell migration (25), and cell invasion (26) were performed as described previously. All *in vitro* assays were performed in triplicate, and all of the experiments were repeated at least three times, with similar results obtained for each replicate. (#P < 0.05, *P < 0.01).

Western blotting, real-time quantitative PCR, immunofluorescence, luciferase reporter assay, pulse-chase assay.

The protein and mRNA expression levels of MDM2 and other molecules were determined by Western blotting and real-time quantitative PCR, respectively (27). The primer sequences

used for the amplification of genes were as follows: MDM2 sense, 5'-ATCATCGGACTCAGGTACA-3'; MDM2 antisense, 5'-GTCAGCTAAGGAAATTCAGG-3'; CCNE1 sense, 5'-TCT GGA TTG GTT AAT GGA GGT-3'; CCNE1 antisense, 5'-TGG TGC AAC TTT GGA GGA-3'; GAPDH sense, 5'-GGAGTCCACTGGCGTCTTCAC-3'; GAPDH antisense, 5'-GAGGCATTGCTGATGATCTTGAGG-3'. Immunofluorescence staining was performed to determine the expression and location of the MDM2 protein in the cells (24). The cytoplasmic and nuclear fractions were extracted from MDA242-treated cells using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). The *MDM2* promoter activity was determined using a luciferase reporter assay system (Promega, Madison, WI) (23). Briefly, cells were transfected with *MDM2* P1 or P2 (full-length, deleted, or mutated) luciferase plasmids with Renilla luciferase reporter as an internal control for 12 h, followed by treatment with various concentrations of MA242 for 24 h. The degradation rates of proteins and mRNAs were examined using pulse-chase assays (27).

Electrophoretic mobility shift assay (EMSA).

The cells were treated with MA242 in the presence or absence of ionomycin (4 μ M), an activator of calcineurin, for 6 h, then the nuclear fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL) (23). The prepared nuclear extracts were preincubated with 1 μ g of poly-(dI:dC) (Thermo Scientific, Rockford, IL) and then reacted with a biotin-labeled MDM2 probe at room temperature for 30 min. The sequences of the biotin-labeled probes were: MDM2 forward, 5'-GCAGTTGACTCAGCTTTTCTCTTGAGCTGGTCAAGTTCA-3' and MDM2 reverse, 5'-TGAACCTTGACCAGCTCAAGAGGAAAAGCTGAGTCAACCTGC-3'.

Chromatin immunoprecipitation (ChIP) assay.

The cells were treated with DMSO as a vehicle control or with MA242 for 6 h, then the ChIP assay was performed as reported previously (23). The specific primer pairs used were: 5'-CCCCCGTGACCTTTACCTG-3' and 5'-AGCCTTTGTGCGGTTTCGTG-3' for qualitative or quantitative PCR amplification of the responsive element of the *MDM2* promoter.

Development and treatment of orthotopic pancreatic cancer models.

The animal protocols were approved by the Institutional Animal Use and Care Committee. Female 4–6-week-old athymic nude mice (nu/nu, 4–6 weeks) were obtained from Charles River Laboratories International, Inc. (Wilmington, MA). Two pancreatic cancer cell lines, AsPC-1-Luc and Panc-1-Luc, were employed in this study (24). Briefly, 30 μ L of AsPC-1-Luc or Panc-1-Luc cell solution (1×10^6 cells in PBS) was injected into the head of the pancreas. MA242 was dissolved in PEG400:ethanol:saline (57.1:14.3:28.6, v/v/v). The treatment was carried out in the following way: The control group received the vehicle only. For AsPC-1 tumor-bearing mice, MA242 was administered by intraperitoneal injection at a dose of 10 mg/kg/d, 5 d/wk for three weeks. For Panc-1 tumor-bearing mice, MA242 was administered by intraperitoneal injection at a dose of 2.5 or 5 mg/kg/d, 5 d/wk for five weeks. Gemcitabine (Selleckchem, Houston, TX) was administered at a dose of 20 mg/kg on days 1, 3 and 5 every week for two weeks. The combination group received MA242 at a

dose of 5 mg/kg, 5 d/wk for five weeks and gemcitabine at a dose of 20 mg/kg on days 1, 3 and 5 every week for two weeks. At the end of the experiments, all mice were examined for tumor metastasis to various organs, tumors and various tissues were removed for Western blotting and pathology evaluation (24, 25).

Immunohistochemistry, TUNEL staining, and hematoxylin and eosin (H&E) staining.

Briefly, tumors and various tissues were removed from mice, fixed in 10% formalin, and embedded in paraffin. The tumor and tissue sections (5 μ m thick) were then prepared, deparaffinized, rehydrated, and washed (24, 25). The immunohistochemical staining of target proteins was performed using biotinylated antibodies and then the sections were counterstained with hematoxylin, mounted, and analyzed. The *in vivo* apoptosis was measured using a TUNEL staining kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions (24, 25).

Statistical analysis.

The data were analyzed using the Prism software program version 6 (Graph Pad software Inc., San Diego, CA, USA). Two-sided *t*-tests were used for comparisons between two groups. All data are expressed as the means $\pm$ SEM from at least three independent experiments. $P < 0.05$ was considered to be statistically significant.

Results

MA242 is a potent and selective dual inhibitor of MDM2 and NFAT1, independent of p53.

Thus far, there have been no specific MDM2 and NFAT1 dual inhibitors available. We were motivated to identify bifunctional small molecules that could bind to the both MDM2 and NFAT1, promoting MDM2 degradation and inhibiting NFAT1-mediated MDM2 transcription, respectively. By employing high-throughput virtual and cell-based screening assays, we discovered a class of makaluvamine analogs with the desired properties. To improve the activity and specificity of makaluvamines, we further designed and synthesized a class of makaluvamine analogs (data not shown). One of these analogs, MA242 (Figure 1A), was identified as a potent and selective MDM2 and NFAT1 dual inhibitor in a molecular modeling study, Biacore assay, and pull-down assays (data not shown). As shown in Supplementary Figure S1A-B, at a concentration of 0.1 μ M, MA242 induced a significant reduction of the MDM2 and NFAT1 expression in Panc-1 cells, indicating that it was the most potent of the new compounds. Similar results were obtained in other pancreatic cancer cell lines (data not shown).

To assess its activity and selectivity, MA242 was examined for effects on cell viability in a normal pancreatic HPDE cell line (p53 wild-type (wt)) and a panel of MDM2-overexpressing pancreatic cancer cell lines, including HPAC (p53 wt), Panc-1 (p53 mutant (mt)), AsPC-1 (p53 null), Mia-Paca-2 (p53 mt) and BxPC-3 (p53 mt) (28). As shown in Figure 1B, MA242 significantly inhibited pancreatic cancer cell growth, with IC₅₀ values ranging from 0.1 to 0.4 μ M, regardless of the p53 status of the cells. However, MA242 showed minimal effects on the growth of normal HPDE cells (IC₅₀ = 5.81 μ M), indicating that MA242 had selective effects against cancer cells. MA242 was further evaluated for *in*

vitro anticancer activity and the specificity of its inhibition of MDM2 and NFAT1 in pancreatic cancer cells with different p53 backgrounds, including the HPAC, Panc-1 and AsPC-1 cell lines. MA242 effectively inhibited the cell colony formation (Figure 1C), slightly arrested cells at G2/M phase (Figure 1D), and induced apoptosis (Figure 1E) in all three cell lines in a concentration-dependent manner, regardless of the p53 status of the cells, indicating that apoptosis was the major regulatory mechanism for MA242-induced cell death. In addition, MA242 significantly decreased the MDM2 and NFAT1 protein levels at a low concentration in all three cell lines (Figure 1F). However, MA242 did not affect the protein expression levels of cyclin E in all three cell lines, indicating that the compound did not nonspecifically affect other inducible proteins. We also observed that when MDM2 was inhibited by MA242, there was activation of p53 signaling in HPAC (p53 wt) cells, but no effect on the expression levels of p53 in the Panc-1 (p53 mut) and AsPC-1 (p53 null) cells. MA242 also increased the expression levels of p21 and cleaved PARP in a p53-independent manner.

MA242 suppresses orthotopic pancreatic tumor growth in vivo, independent of p53.

To confirm that MA242 exerts p53-independent anti-pancreatic cancer activity *in vivo*, we next examined its efficacy in Panc-1 and AsPC-1 orthotopic tumor models. As shown in Figure 2A, the nude mice bearing Panc-1 orthotopic tumors were treated with vehicle or MA242 (2.5 and 5 mg/kg/day, 5 days/week) by i.p injection for five weeks, resulting in 56.1% and 82.5% inhibition of tumor growth, respectively (Figure 2A1-A2). In the AsPC-1 orthotopic model, mice were treated with MA242 at 10 mg/kg/day, 5 days/week for three weeks. MA242 significantly suppressed the growth of AsPC-1 orthotopic tumors by 89.5% ($P < 0.01$) compared with the tumors in control animals (Figure 2B1-B2). MA242 treatment led to almost complete tumor regression in MD242-treated mice in both models. Importantly, there were no significant differences in the average body weights between the vehicle- and MA242-treated mice in either of the models, suggesting that MA242 did not have significant host toxicity at these effective doses (Figures 2A3 and 2B3). At the end of the experiments, the major organs (liver, lungs, kidneys, spleen, heart and brain) were dissected from mice bearing AsPC-1 orthotopic tumors for histological examinations, and no tumor metastasis or organ abnormalities were observed in control or treatment groups (Supplementary Figure S2), confirming the efficacy and safety of the treatment.

MA242 inhibits MDM2 and NFAT1 in vivo.

To examine the effects of MA242 on MDM2 and NFAT1 *in vivo*, we evaluated the protein expression levels of MDM2 and NFAT1 in both Panc-1 and AsPC-1 orthotopic tumors. As shown in Figure 2C, the immunohistochemical staining of tumor tissues indicated that the MDM2 and NFAT1 expression levels were significantly reduced in MA242-treated tumors. TUNEL staining of the tumor tissues demonstrated that there was a significant increase in apoptosis in the MA242-treated tumors, compared with that observed in vehicle-treated tumors. These results were confirmed by a Western blotting analysis (Figure 2D). Consistent with the *in vitro* results, MA242 decreased the expression of MDM2 and NFAT1, and increased the expression levels of p21 and cleaved-PARP in both AsPC-1 and Panc-1 orthotopic tumors, compared with the tumors from control animals.

MA242 sensitizes pancreatic cancer cells to gemcitabine treatment *in vitro* and *in vivo*.

Overcoming gemcitabine resistance in pancreatic cancer cells remains a major challenge to the effective treatment of the disease (29). MA242 was further examined for its effects on the sensitivity of gemcitabine-resistant HPAC, Panc-1, and AsPC-1 cell lines (30) to gemcitabine. As shown in Figure 3A, treatment with MA242 at a low concentration (0.05 μ M) significantly increased the gemcitabine sensitivity in all three cell lines, with remarkably reduced IC₅₀ values for gemcitabine in all of these cell lines. Furthermore, gemcitabine induced more apoptosis in pancreatic cancer cells that were treated with MA242 than was observed in the vehicle-treated cells (Figure 3B).

To determine whether MA242 sensitizes pancreatic cancer cells to chemotherapy *in vivo*, the effects of gemcitabine (20 mg/kg, i.p. injection on days 1, 3 and 5/week for two weeks) were evaluated alone or in combination with MA242 (5 mg/kg) in the Panc-1 orthotopic tumor-bearing mice. As shown in Figure 3C1-C2, gemcitabine alone exerted only moderate inhibitory growth effects, with tumor growth inhibited by about 47.8%, while the combination of gemcitabine with MA242 led to a complete tumor regression in almost all of the mice (94.1% inhibition). Of note, there were no significant changes in the average body weight in the MA242 and gemcitabine combination-treated mice compared to control mice (Figure 3C3), indicating that MA242 and gemcitabine combination was safe at the effective therapeutic doses.

MA242 prevents pancreatic cancer metastasis *in vitro* and *in vivo*.

The propensity of pancreatic cancer for early metastasis, with frequent involvement of the peritoneum, liver and lungs, is one the major reasons for the low five-year survival rate (~6%) in patients (31). Therefore, we next examined the effects of MA242 on cell migration and invasion with Panc-1 and AsPC-1 cell lines, which are invasive and metastatic (24). As shown in Figure 4A, the Panc-1 and AsPC-1 cells both migrated into the entire wounded area by 48 h, whereas treatment with MA242 at low concentrations (0.05 and 0.1 μ M) significantly inhibited the cell migration. Similarly, at a concentration of 0.1 μ M, MA242 reduced the invasion of Panc-1 and AsPC-1 cells by 75.8% and 77.0%, respectively, compared to the control (Figure 4B).

MA242 was further examined for effects on metastasis in mice bearing Panc-1 orthotopic tumors. Necropsy showed that 10 of 11 mice treated with the vehicle developed metastatic lesions in the peritoneum, whereas the incidence of peritoneal dissemination in the mice treated with 2.5 and 5 mg/kg/d MA242 was 4/11 and 3/11, respectively (Figure 4C-D). Compared with the vehicle-treated mice, MA242 treatment also significantly inhibited the metastasis to the liver and lungs in a dose-dependent manner (Figures 4C, 4D, and 4E). Of note, 5, 5, and 1 of 12 mice treated with gemcitabine were found to have metastatic lesions in the peritoneum, liver and lungs, respectively, while only 1 of 11 mice treated with the combination of gemcitabine and MA242 had liver metastasis, and none of these mice had peritoneal or lung metastases (Figures 4C, 4D and 4E). These results were confirmed by histopathological examinations of the liver and lung tissues from mice in the control and treatment groups (Supplementary Figure S3A). The metastatic cells in livers and lungs were further confirmed using IHC staining of specific pancreatic cancer biomarkers CA19-9 and

cytokeratin 7 (Supplementary Figure S3A). No metastasis or abnormalities were found in any other major organs (kidneys, spleen, heart and brain) from the treated mice, indicating the absence of any overt host toxicity (Supplementary Figure S3A).

MA242 induces MDM2 self-ubiquitination.

We next examined MA242's effects on the turnover of the MDM2 protein in the HPAC and Panc-1 cell lines. As shown in Figure 5A, MA242 shortened the half-life of MDM2 in both cell lines. This protected wild-type p53 from MDM2-mediated degradation in the HPAC cells, but there was no significant effect of MA242 on mutant p53 in the Panc-1 cells. Further, the MA242-induced MDM2 inhibition was reduced by treatment of the cells with a proteasome inhibitor, MG-132 (Figure 5B), indicating that MA242 promoted the proteasomal degradation of MDM2. These results were confirmed by MDM2 ubiquitination assays in both HPAC and Panc-1 cells (Figure 5C).

To determine if MA242 specifically induces MDM2 self-ubiquitination, its effects on the expression of wild-type MDM2 and a MDM2 mutant (C464A) lacking ubiquitin E3 ligase activity were examined. As shown in Figure 5D, MA242 induced the degradation of wild-type MDM2, but it had no significant effect on mutant MDM2. An immunofluorescence study was then performed, which showed that the degradation of both nuclear and cytoplasmic MDM2 was increased by MA242 treatment (Figure 5E). The similar results were confirmed by Western blot analysis (Figure 5F).

MA242 represses NFAT1-mediated MDM2 transcription.

As shown in Figure 6A, MA242 concentration-dependently inhibited *MDM2* mRNA expression; however, it did not affect the stability of the *MDM2* mRNA (Supplementary Figure S4A). The compound also did not affect the mRNA expression of *CCNE1*, indicating a specificity of MA242's inhibitory effects on *MDM2* mRNA expression (data not shown). Further, MA242 inhibited the *MDM2* P2 promoter activity in a concentration-dependent manner, but not *MDM2* P1 promoter activity (Figure 6B).

Several transcription factors have been reported to regulate *MDM2* transcription via the P2 promoter (32). To determine which transcription factor was involved in the MA242-induced inhibition of *MDM2* transcription, various deletions (Supplementary Figure S4A) and site mutations of the *MDM2* P2 promoter were transfected into HPAC and Panc-1 cells, followed by treatment with MA242. The results showed that the shortest deletion, Luc 03, retained the responsiveness to MA242 (Figures 6C and Supplementary Figure S4A). Next, we made site mutations of several transcription factor binding sites in this segment, including those for MEF2, NFAT1, AP1 and ETS. The results showed that mutation of the NFAT1 site significantly reduced the effects of MA242 on the P2 promoter activity, indicating that MA242 could inhibit NFAT1-mediated *MDM2* transcription (Figures 6D and Supplementary Figure S4A). The results were confirmed using *MDM2* P2 promoters with double mutations (AP1-ETSα) and triple mutations (AP1-ETSα-NFAT) (Figures 6E and Supplementary Figure S4A).

Further studies were performed to determine how MA242 inhibits NFAT1-mediated *MDM2* transcription. Since NFAT1 activates *MDM2* transcription via direct binding to the *MDM2*

P2 promoter (23), EMSA and ChIP assays were utilized to examine the effects of MA242 on the NFAT1-*MDM2* P2 promoter complex. As shown in Figure 6F, MA242 inhibited the ionomycin (ION, an activator of the calcineurin-NFAT pathway)-enhanced NFAT1-*MDM2* P2 promoter binding in HPAC and Panc-1 cells. Similar results were further confirmed by the ChIP assay (Figure 6G).

The MA242-induced anticancer activities depend on MDM2 inhibition.

To demonstrate the importance of MDM2 for the sensitivity of the pancreatic cancer cells to MA242 treatment, MDM2 knockdown (KD) experiments using the CRISPR/Cas9 technique were performed in Panc-1 cells. As shown in Figure 7A, the MDM2 KD stable cell line appeared approximately 92% knockdown of MDM2 protein expression. Treatment of the parental cells with MA242 inhibited the expression of NFAT1 and induced the expression of cleaved-PARP in a concentration-dependent manner. Compared with these control Panc-1 cells, MDM2 KD itself caused 20% inhibition of cell growth (Figure 7B) and 2-fold increase in apoptosis (Figure 7C). In addition, the MDM2 KD cells showed a decreased response to MA242, leading to decreased effects of MA242 on the growth and apoptosis of the cells (Figures 7B-C). Similar results were obtained in HPAC and Panc-1 cells with siRNA-mediated MDM2 KD (Supplementary Figure S5A-F). To demonstrate that MA242-induced MDM2 degradation causes apoptosis but MA242-induced apoptosis does not cause MDM2 degradation, we further determined the effects of MA242 on MDM2 expression and cell apoptosis in the presence or absence of an apoptosis inhibitor z-VAD-FMK. As shown in Supplementary Figure S6A-B, z-VAD-FMK significantly reduced MA242-induced cell apoptosis but it did not affect MA242's inhibitory effects on MDM2, indicating that MA242 caused MDM2 degradation, leading to cell apoptosis.

Discussion

The aim of the present study was to develop a safe and effective dual MDM2 and NFAT1 inhibitor for pancreatic cancer therapy. MDM2 has been demonstrated to be a 'druggable' target since it was discovered to be the major negative regulator of the p53 tumor suppressor (7–12), and a variety of strategies have been developed to inhibit its expression and/or functions. Several MDM2 inhibitors are currently in clinical trials (14), but these inhibition strategies have proven to be challenging for several reasons. First, wild-type p53 is necessary for all inhibitors that target the MDM2-p53 interaction, but p53 mutation is a common genetic event in human cancers. Second, other than the p53 binding site, it has proven difficult to identify other active binding sites for small molecules on MDM2. Third, specific inhibition of MDM2 expression and/or functions by these inhibitors leads to a hyperactive compensation of MDM2 through other pathways, such as p53-induced feedback-mediated increases in MDM2. Therefore, the therapeutic efficacy of the current MDM2 inhibitors will likely be limited in human cancers, especially in those with p53 deficiency. New and more effective strategies for targeting MDM2 are needed.

It has also been shown that NFAT1 is overexpressed in malignant pancreatic ductal adenocarcinomas compared with normal adjacent tissues, and is associated with advanced stages of the disease (21). Therefore, we and others have proposed NFAT as a target for

cancer therapy. Indeed, a few calcium channel blockers (e.g., CsA and tacrolimus) have been found to inhibit NFAT1 activity and showed promising anticancer activity in the preclinical setting that was postulated to be related to effects on calcineurin signaling. However, due to the lack of specificity of these drugs, patients on long-term immunosuppressive treatments actually exhibit increased rates of cancer incidence, neuro- and nephrotoxicity, as well as cardiovascular and diabetic complications (20). Therefore, new treatment strategies that specifically inhibit NFAT1 activity are needed.

Most of the previously developed anticancer SMIs specifically target one gene in one pathway. However, due to the presence of pathway convergence and crosstalk, the exclusive inhibition of one target often leads to the hyperactivation of other pathways, as well as limited efficacy in clinical trials. In the present study, we have identified MA242 as a new dual inhibitor of MDM2 and NFAT1 based on virtual structural screening and physical-chemistry evaluations. MA242 is distinct from previous single-pathway inhibitors; it exhibits dual inhibition of both MDM2 and NFAT1, leading to a profound reduction of both the MDM2 and NFAT1 levels in pancreatic cancer cells, regardless of the p53 status. Our results demonstrated that MA242 increased MDM2 self-ubiquitination and promoted its proteasomal degradation. In addition, MA242 enhanced NFAT1 protein degradation, resulting in the repression of *MDM2* transcription. Most importantly, the MA242-induced anticancer activity is dependent on MDM2 inhibition. In the present study, in order to validate that MDM2 is the major target of MA242, we used both CRISPR/Cas9 gene editing and siRNA techniques. We confirmed that knockdown of MDM2 was toxic to the cells and demonstrated that knockdown of MDM2 resulted in decreased effectiveness of the compound MA242, supporting the importance of MDM2 inhibition in the MA242-induced anti-pancreatic cancer activities.

Considering that resistance to conventional therapies and early metastasis are the major causes of pancreatic cancer mortality, and p53 mutations were largely responsible for the limited efficacy of MDM2 inhibitors in previous studies (29, 33), we used a variety of pancreatic cancer models to evaluate the translational potential of MA242. First, normal pancreatic cells and a panel of pancreatic cancer cell lines with various backgrounds of p53 (wt, mt, and null) were used to demonstrate the cancer cell selectivity and MDM2 inhibitory effects of MA242. HPAC cells showed different responses to MA242 treatment in MTT assay and colony formation assay, which indicated that MA242 might mainly inhibit the cell proliferation but not cell metabolic activity. In addition, the p53-induced feedback increase in MDM2 could be partially responsible for this discrepancy. Second, the *in vivo* efficacy and safety of MA242, and its p53-independent effects, were examined in both AsPC-1 and Panc-1 orthotopic mouse models. We first demonstrated the *in vivo* efficacy of MA242 in a Panc1 orthotopic model at 2.5 and 5 mg/kg because we did not observe any host toxicity at these doses in our initial safety studies (data not shown). To further demonstrate the *in vivo* efficacy and safety of MA242, we assessed the effects of the compound at a higher dose (10 mg/kg) in an AsPC1 orthotopic model. These results indicated that MA242 at 10 mg/kg is a safe and effective dose for treating human pancreatic cancer *in vivo*. Third, the effects of MA242 on cell migration and invasion were examined in pancreatic cancer cell lines with invasive and metastatic properties; the *in vivo* efficacy against tumor metastasis was also evaluated. Fourth, three gemcitabine-resistant cell lines were utilized to demonstrate the

MA242-induced chemosensitization of pancreatic cancer cells to gemcitabine *in vitro*, while one of these cell lines was further used to develop an orthotopic mouse model for *in vivo* evaluation. Although gemcitabine is a first-line treatment for patients with locally advanced or metastatic pancreatic cancer, a large number of patients with pancreatic cancer do not respond to gemcitabine due to the high levels of intrinsic and acquired chemoresistance. Gemcitabine can interrupt DNA synthesis and then cause cell death by apoptosis (34). Several signaling pathways playing critical roles in the development of gemcitabine chemoresistance have been reported. Genetic or epigenetic alterations regulating gemcitabine-induced apoptosis, such as mitogen activated protein kinase p38 (p38MAPK), p53, histone deacetylase (HDAC), and ceramide, appear to impact pancreatic cancer chemosensitivity directly or indirectly (35–38). In the present study, we observed a p53-independent apoptosis induction on the treatment of the cells with the gemcitabine treatment, which was significantly altered by the combination of MA242. As examples of the potential p53-dependent effects of combination treatment, p53 activation by MA242 and gemcitabine stimulates a wide network of signals that act through both extrinsic and intrinsic apoptotic pathways, including the activation of a Caspase cascade and the shifting the balance in the Bcl-2 family towards the pro-apoptotic members. In p53 non-functional cells, it is possible that MA242 and gemcitabine treatment causes other well-known apoptosis mediators directly. Gemcitabine induces the activation of acidic sphingomyelinase (SMase) and increases the production of ceramide, resulting in apoptosis induction (38). MA242 may active or physically binds to other MDM2-related apoptosis mediators such as p73 and FOXO3a (39), leading to subsequent induction of apoptosis. In the present report, our combination therapy significantly improved the efficacy of gemcitabine alone, demonstrating chemosensitization of gemcitabine-resistant cells *in vitro* and *in vivo*. However, the mechanisms underlying MA242-mediated chemosensitization are still not fully understood. Further studies are needed to explore the molecular mechanisms by which MA242 increases chemosensitization.

In summary, MA242 is a potent and selective dual inhibitor of MDM2 and NFAT1 with distinct mechanisms of action, compared to the existing MDM2 or NFAT1 inhibitors. We have also shown that MA242 has promising efficacy both alone and in combination with gemcitabine in several preclinical models of pancreatic cancer. Our data suggest that the pharmacological inhibition of both MDM2 and NFAT1 is a promising strategy for the treatment of this disease, even in tumors lacking functional p53. MA242 warrants further evaluation as new therapeutic option for pancreatic cancer to be used alone and/or in combination with gemcitabine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Findings suggest that pharmacological inhibition of both MDM2 and NFAT1 is a promising strategy for the treatment of pancreatic cancer, even in tumors lacking functional p53.

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Figure 1. MA242 selectively induces pancreatic cancer cell death and inhibits MDM2 and NFAT1 expression, independent of p53.

(A) The chemical structure of MA242. Cells were exposed to various concentrations of MA242 for (B) 72 h for the cell viability assay to determine the 50% inhibitory concentration (IC₅₀) values; (C) 24 h for the colony formation assay; (D) 24 h for the cell cycle distribution assay; (E) 48 h for the apoptosis assay; and (F) 24 h for studies of the expression of MDM2, NFAT1 and other proteins related to cell cycle progression and apoptosis in human pancreatic cancer cells. (* $P < 0.05$; # $P < 0.01$).

Figure 2. MA242 suppresses orthotopic pancreatic tumor growth and inhibits MDM2 and NFAT1 expression, independent of p53.

Panc-1-Luc and AsPC-1-Luc cells were implanted orthotopically into the pancreata of nude mice. Mice were treated with MA242 by i.p. injection at doses of 2.5 and 5 mg/kg/d, 5 d/wk for five weeks in the Panc-1 model, and 10 mg/kg/d, 5 d/wk for three weeks in the AsPC-1 model. The luciferase signals in the mice bearing Panc-1 (A1) or AsPC-1 (B1) orthotopic tumors were detected, and images were obtained using an IVIS *in vivo* imaging system. (A2 & B2) At the termination of the experiments, the average tumor mass (determined by the detected photons/sec) of the MA242-treated mice was compared with that of the control mice. (A3 & B3) The mice were monitored for changes in body weight as a surrogate marker for toxicity. At the end of the experiments, the orthotopic tumors were carefully removed and analyzed by (C) immunohistochemical and TUNEL staining (all images represent serial sections; scale bar, 20 μ m) and (D) Western blotting. (# $P < 0.01$)

Figure 3. MA242 sensitizes pancreatic cancer cells to gemcitabine *in vitro* and *in vivo*, independent of p53.

HPAC, Panc-1, and AsPC-1 cells were treated with MA242 (0.05 μ M) and various concentrations of gemcitabine (Gem, 0, 10, 25, 50, and 100 nM) for (A) 72 h for the cell viability assays and to determine the IC₅₀ values or (B) 48 h for the apoptosis assays. All assays were performed in triplicate and repeated at least three times. Mice bearing orthotopic Panc-1 tumors were treated with vehicle, gemcitabine (Gem, 20 mg/kg/d, 3 d/wk for 2 weeks, i.p.), or a combination of MA242 (5 mg/kg, 5 d/wk for 5 weeks, i.p.) and Gem (20 mg/kg). (C) The luciferase signals in the mice were detected, and images were obtained using an IVIS *in vivo* imaging system. At the termination of the experiments, the mean tumor masses in the treatment groups were compared with that in the control group. (D) The

mice were also monitored for changes in body weight as a surrogate marker for toxicity. (* $P < 0.05$; # $P < 0.01$)

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Figure 4. MA242 inhibits cell metastasis *in vitro* and *in vivo*, independent of p53.

(A) Confluent monolayers of Panc-1 and AsPC-1 cells were scratched with a 1-mL pipette tip at experimental time zero, and then were treated with MA242 (0.05 and 0.1 μ M). The wells were photographed at different time points after the scratch was made. (B) Panc-1 and AsPC-1 cells were exposed to MA242 (0.05 and 0.1 μ M), then the cell invasion was determined by the Transwell invasion assay. (C) At the termination of the experiments, the mice bearing Panc-1 orthotopic tumors were examined for tumor metastasis to various organs. The numbers of mice with metastasis to the peritoneum, liver, and lungs were counted. (D) Representative images showing Panc-1 tumor metastasis to various organs. (E) The livers and lungs were carefully removed from the mice and imaged to detect metastatic lesions. (* $P < 0.05$; # $P < 0.01$)

Figure 5. MA242 promotes MDM2 self-ubiquitination and degradation, independent of p53.

(A) HPAC and Panc-1 cells were treated with DMSO or MA242 (0.2 μ M) for 24 h, followed by exposure to a protein synthesis inhibitor, cycloheximide (CHX, 15 μ g/mL). The protein expression levels of MDM2 and p53 were detected by Western blotting at the indicated times after exposure to CHX. (B) Cells were treated with DMSO or MA242 (0.2 μ M) for 24 h, then were exposed to MG-132 (25 μ M), a proteasome inhibitor, for an additional 6 h. The protein levels of MDM2 and p53 were detected by Western blotting. (C) Cells were co-transfected with MDM2 and ubiquitin plasmids, followed by treatment with DMSO or

MA242 for 24 h. Cell lysates were subjected to immunoprecipitation with an anti-MDM2 antibody. The ubiquitinated MDM2 was detected using an anti-ubiquitin antibody. (D) Cells were transfected with a wild-type MDM2 plasmid or a mutant MDM2 plasmid (C464A) without E3 ligase activity, followed by exposure to MA242 for 24 h, and the MDM2 levels were detected by Western blotting. (E) Cells were treated with DMSO or MA242 (0.2 μ M) for 6 h, followed by immunofluorescence detection (scale bar, 5 μ m). β -actin and DAPI were used as internal references. (F) Cells were treated with DMSO or MA242 for 24 h, and the cytoplasmic and nuclear fractions were extracted from the cells using the NE-PER nuclear and cytoplasmic extraction kit. The MDM2 distribution in the cytoplasm and nucleus was examined by Western blotting. α -Tubulin and Lamin B were used as internal references, respectively.

Figure 6. MA242 inhibits NFAT1-mediated *MDM2* transcription.

(A) HPAC and Panc-1 cells were treated with MA242 for 24 h. The relative levels of *MDM2* mRNA were determined by quantitative RT-PCR and normalized to those of GAPDH mRNA. (B) Cells were transfected with either *MDM2* P1 promoter luciferase (*MDM2* P1-Luc) or *MDM2* P2 promoter luciferase (*MDM2* P2-Luc) for 12 h. The transfected cells were exposed to various concentrations of MA242 for 24 h, then the luciferase activity was measured. Panc-1 cells were transfected with full length or (C) deleted *MDM2* P2 promoters, (D) site-mutated *MDM2* P2 promoters, and (E) *MDM2* P2 promoters with a double or triple mutation for 12 h, followed by treatment with MA242 before the luciferase activity was measured. (F) HPAC and Panc-1 cells were treated with 0.2 μ M MA242 for 24 h in the presence or absence of ionomycin (ION, 4 μ M). The nuclear proteins were extracted and incubated with an *MDM2* probe. (G) Cells were treated with 0.2 μ M MA242 for 24 h, then the cell lysates were immunoprecipitated with anti-NFAT1 antibodies or IgG. The DNA

bound to the endogenous NFAT1 was eluted and quantified by PCR using primers specific for the *MDM2* P2 promoter. (* $P < 0.05$, # $P < 0.01$, and “ns” denotes “not significant”)

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Figure 7. CRISPR/Cas9-mediated MDM2 knockdown blocks MA242's activity, independent of p53.

Parental Panc-1 cells and Panc-1 cells with MDM2 knockdown by a CRISPR/Cas9 system were treated with various concentrations of MA242 for (A) 24 h for assessment of the protein expression of MDM2; (B) 72 h for the cell viability assay; and (C) 48 h for the apoptosis assay. (* $P < 0.05$ and # $P < 0.01$)